PHOSPHATE TRANSPORT AND ATP SYNTHESIS IN YEAST MITOCHONDRIA

Effect of a new inhibitor: the tribenzylphosphate

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1 Introduction

We have shown that phosphate transport in yeast mitochondria follows two mechanisms a well-defined carrier-mediated process (with a relatively low K_T) and a like-diffusion process [1,2], both were inhibited by mersalyl (see [3,4]) The carrier-mediated transport was dependent on the mitoribosomal protein synthesis [2] and the implication of a P_i-binding proteolipid, having also a mitoribosomal origin, was therefore expected [5]

The existence of these two systems for transport allowed us to define their role in the metabolic pathway of P_i in mitochondria Here we describe the action of a new inhibitor of both P_i-transport and ATP synthesis Our results are consistent with the idea that the carrier-mediated P_i-transport is specifically implicated in the oxidative phosphorylation process as suggested for mammalian mitochondria [6]

2 Materials and methods

Cells of the diploid yeast strain Yeast Foam were grown aerobically with 2% galactose as carbon source, and mitochondria were prepared as in [4,7] Mitochondrial protein concentration was estimated by the biuret method Oxygen uptake was measured with a Clark oxygen electrode (Gilson) at 28°C in 3 ml of the following basal medium 10 mM Tris–maleate, 0 65 M mannitol, 0 3% BSA (pH 6 7) In order to maintain the structure of the mitochondria the basal medium was free of KCl [8] The mitochondrial volume spaces were determined as in [4] and the ΔpH by the distribution of [^{3}H]acetate between the intra- and extra-matricial spaces [9] Phosphate transport was measured as in [2] $^{32}P_i$ incorporation in adenylic nucleotides was measured as follows mitochondria (1 5 mg protein) were suspended in 3 ml of the basal medium containing 2 mM ADP and the respiratory substrate at 28°C (for particular conditions see figure legends), the reaction was started by addition of 1 mM $^{32}P_i$ and stopped at a defined time by 0 3 M trichloroacetic acid After centrifugation, 0 5 ml Norit (100 mg/ml) was added into 0 4 ml of the supernatant The suspension was thoroughly mixed, then filtered through a disc of paper (Whatman no 1), washed with 0 2 M potassium phosphate and then with distilled water The filter was dried and counted in a gas-flow counter (Nuclear-Chicago)

Tribenzylphosphate was purchased from Sigma Chemicals Co

3 Results

Figure 1A shows that TBP inhibited completely the respiration rate stimulated by ADP for mitochondria oxidizing ethanol (at concentrations below 0 2 mM), the controlled respiration remained uninhibited On α-ketoglutarate, TBP inhibited <60% state 3 respiration (fig 1B) This value equals the inhibition obtained by oligomycin, this fact can be explained by

Abbreviations TBP, tribenzylphosphate, P_i, inorganic phosphate, AdN, adenylic nucleotides, CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

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a partial control of the respiration at the substrate level [10,11]; thus, an apparent respiration control would not prove the existence of coupled respiration for mitochondria oxidizing α-ketoglutarate. The uncoupled respiration (in the presence of CCCP) was not inhibited by TBP either with ethanol or α-ketoglutarate as substrates (not shown). The results indicated that the inhibitory action of TBP on state 3 was not due to the inhibition of the electron transport activity but rather to an inhibition of the utilization of the high-energy state.

We had measured directly the effect of TBP on the ATP synthesis at both oxidative and substrate-level phosphorylations. Oxidative phosphorylations were performed in the presence of arsenite, in order to inhibit the substrate level phosphorylations and with ethanol as respiratory substrate. Under such conditions the incorporation of $^{32}P_i$ in adenine nucleotides was equally inhibited by TBP or oligomycin (fig.2A). Substrate-level phosphorylations were followed in the presence of oligomycin and with α-ketoglutarate as substrate. ATP synthesis was completely inhibited by arsenite but not by TBP as shown in fig.2B. These results indicated that TBP acts on the mechanism of energy transduction which operates at the oxidative phosphorylation level. However, TBP did not inhibit the ATP synthetase complex measured by either the oligomycin-sensitive ATPase activity or the ATP–$^{32}P_i$ exchange (unpublished data). Therefore, we studied the effect of TBP on the P_i-transport.

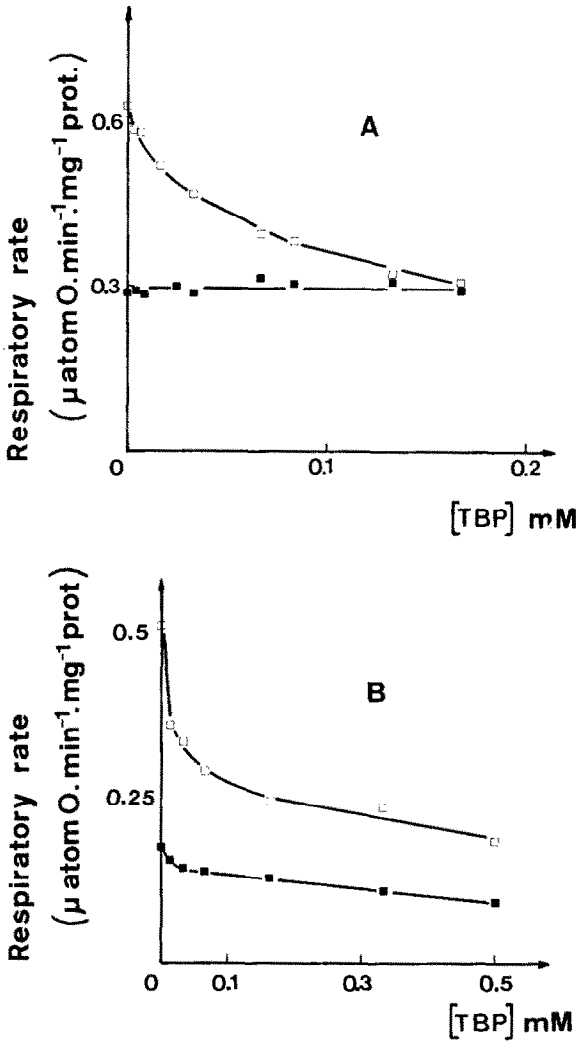

Fig.1. Effect of TBP on the respiratory rate of yeast mitochondria. Mitochondria (1 mg protein) were suspended in the presence of the indicated concentrations of TBP in 3 ml of the basal medium containing 1.3 mM Tris–phosphate at pH 6.7 and 28°C. The respiratory substrates used were: (A) 0.66% ethanol; (B) 3 mM α-ketoglutarate. (■) Respiration without ADP; (□) respiration in the presence of 0.13 mM ADP.

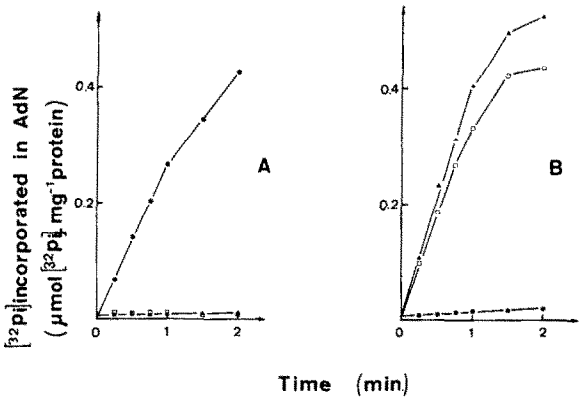

Fig.2. Effect of TBP on the oxidative and the substrate-level phosphorylations. Mitochondria (1.5 mg protein) were suspended of the medium in section 2. The reaction was started by 1 mM $^{32}P_i$ and stopped by 0.3 M trichloroacetic acid additions. (A) Oxidative phosphorylations were measured in the presence of 0.66% ethanol and 9 mM arsenite. (●) Control; (□) 10 μg/ml oligomycin; (▲) 0.17 mM TBP. (B) Substrate-level phosphorylations were measured in the presence of 3 mM α-ketoglutarate and 10 μg/ml oligomycin. (□) control; (●) 9 mM arsenite; (▲) 0.17 mM TBP.

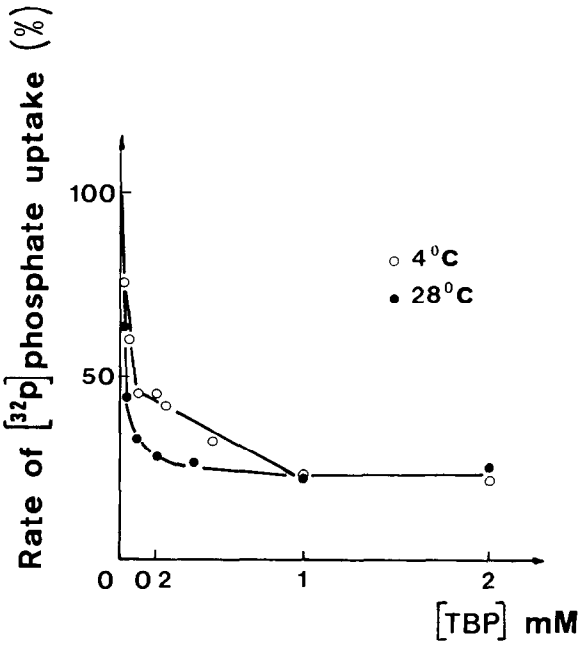

Fig 3 Effect of TBP on the rate of $^{32}P_i$ uptake into mitochondria at two temperatures Mitochondria (2 mg protein) were incubated at 4°C (○) or at 28°C (•) in 0 4 ml of the basal medium containing 0 66% ethanol and 4 μg oligomycin The incorporation was initiated by addition of 0 4 mM $^{32}P_i$ and stopped at different times during the first 10 s by 1 mM mersalyl The radioactivity incorporated in mitochondria was corrected for the extramatricial space TBP was added at indicated concentrations before $^{32}P_i$ The rates of $^{32}P_i$ uptake are given as the % of the rate of $^{32}P_i$ measured in the absence of TBP

Figure 3 shows the effect of TBP on the rate of $^{32}P_i$ uptake in the matricial compartment at two temperatures The maximal inhibition at 28°C was reached for 0 2 mM TBP. The remaining activity (25%) was not sensitive to TBP, but completely inhibited by mersalyl At 4°C the maximum inhibition was obtained for 1 mM TBP Because of the very rapid rates of transport observed, we measured the substrate concentration dependency on the rate of P_i uptake at 4°C at maximum TBP inhibition (1 mM) From the data in [2] it was shown that the double reciprocal plot of phosphate transport versus phosphate concentrations gave an hyperbolic-like curve, indicating the participation of two different uptake systems In the presence of TBP, only one straight line was obtained with any apparent saturation phenomenom (fig 4) This transport does not seem to be due to diffusion, as it was always inhibited by mersalyl However, if this uptake corresponds to a carrier-mediated transport the K_T value would be very high

As shown in fig 5, the concentration dependence

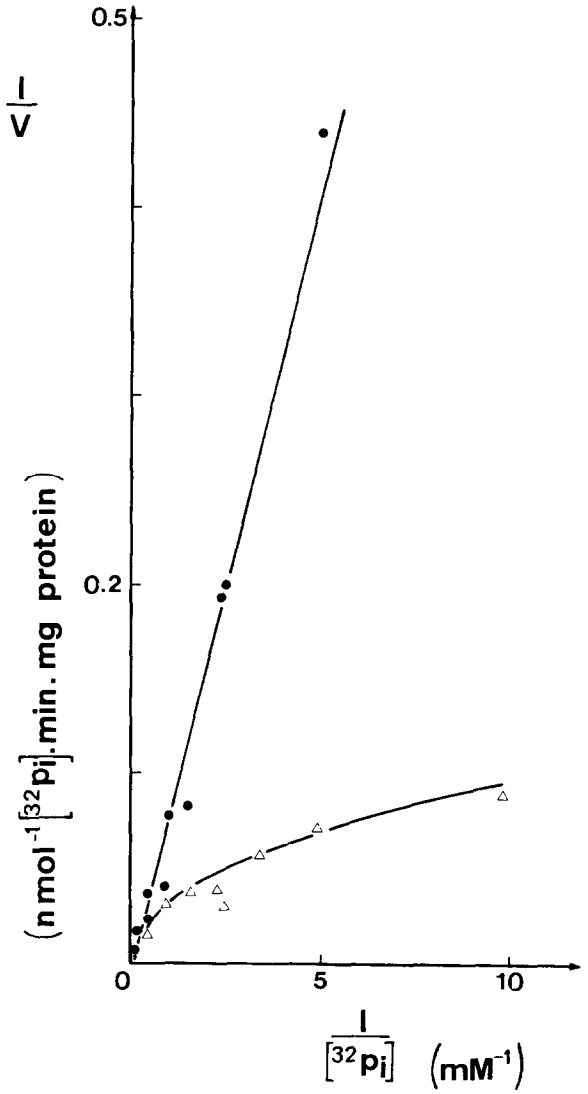

Fig 4 Double reciprocal plot of the $^{32}P_i$ uptake in mitochondria without or in the presence of 1 mM TBP Initial rates of $^{32}P_i$ uptake were measured as described in the legend of fig 3 and at 4°C (△) control, in the presence of 1 mM TBP (•)

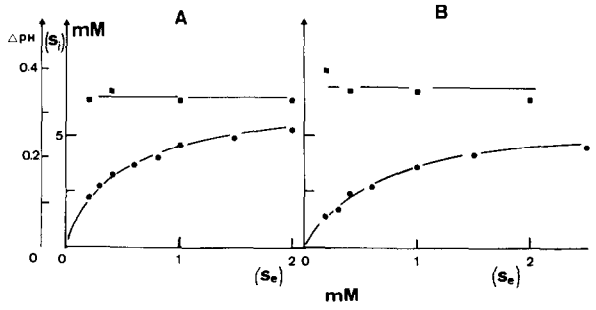

Fig.5. Effect of TBP on the $^{32}P_i$ accumulation and on the transmembranal ΔpH. Mitochondria (2 mg protein) were incubated 10 min in 0.4 ml basal medium containing 0.66% ethanol and different concentrations of $^{32}P_i$ at 4°C and at pH 6.7. In parallel experiments the matricial spaces were measured in the presence of different concentrations of unlabelled P_i. The internal concentration of phosphate (S_i) was plotted against the external one (S_e). The transmembranal ΔpH (■) was measured by the [^{3}H]acetate distribution between the extra- and intra-matricial space (cf. section 2) (A) Control; (B) 1 mM TBP.

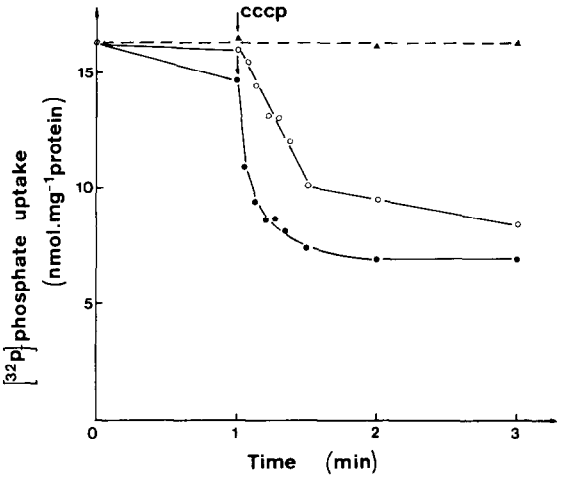

Fig.6. Efflux of phosphate induced by CCCP in mitochondria. Mitochondria were incubated in the basal medium containing 1 mM $^{32}P_i$ for 20 min (corresponding to time 0 of the curve). At this time, 1 mM mersalyl (▲) or 1 mM TBP (●) were added. The efflux of phosphate was induced by 1 μM CCCP. Control (○) without addition before CCCP.

of the steady state accumulation of P_i (measured after 10 min incubation) shows that TBP modified the P_i incorporation slightly but significantly. However, since the transmembranal ΔpH was not dissipated, TBP had no effect on anion accumulation conditions (fig.5). The effect of TBP on incorporation would therefore reflect a modification of the kinetic parameters of the influx and efflux of P_i. When TBP was added to P_i-preloaded mitochondria, it induced a slight efflux of P_i. A rapid efflux of P_i was induced by CCCP and it is shown in fig.6 that the efflux was more rapid in the presence of TBP than in the control.

4. Discussion

TBP inhibits specifically the oligomycin-sensitive ATP synthesis. This inhibition cannot be explained by an effect either on the respiratory chain or on the ATP synthetase complex (defined as a complex catalyzing an oligomycin-sensitive ATP–P_i exchange). TBP is not an uncoupler as it does not dissipate the transmembranal ΔpH. It does not affect the translocase activity as measured by the ADP exchange (not shown here).

Since TBP inhibits ATP synthesis it should affect a system implicated in the transduction of the energy, working between the respiratory chain and the ATPase. The inhibition of ATP synthesis (oligomycin-sensitive) cannot be explained only by inhibition of P_i influx due to TBP since ATP synthesis at the substrate level is not affected by TBP. A qualitative change of the P_i influx could possibly be the fundamental cause of this inhibition. In the presence of TBP the carrier-mediated phosphate transport was completely inhibited whatever being the external P_i concentration. Therefore, two hypothesis could be proposed, which are not contradictory.

1. TBP inhibits the function of a common unit implicated in both ATP synthesis and P_i-transport. This interpretation would be in agreement with the finding [5] where a P_i binding proteolipid was isolated from the OS–ATPase complex.
2. There are two pools of endogenous P_i. The pool (I) depends on the function of the P_i carrier-mediated transport and would be directly implicated in the oxidative phosphorylations. The pool (II) which has no absolute requirement for this carrier function would be implicated in the phosphorylations at the substrate level.

The existence of two compartments for endogenous

phosphate was first postulated in a study on the rate of phosphorylations of AMP and ADP in rat liver mitochondria [12,13] It was proposed that pool (I) of P_i, implicated in the ATP synthesis (oxidative phosphorylation), is more rapidly exchangeable with the external P_i than the pool (II) which is implicated in the ADP synthesis (via the succinyl CoA synthase) Our results agree with this hypothesis

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References

[1] Guérin B , Guerin, M , Napias, C and Rigoulet, M (1977) Biochem Soc Trans 5, 503–506
[2] Rigoulet, M , Guérin, M and Guerin, B (1977) Biochim Biophys Acta 471 280–295
[3] De Chateaubodeau, G , Guérin, M and Guérin, B (1974) FEBS Lett 46, 184–187
[4] Arselin de Chateaubodeau, G , Guérin, M and Guérin, B (1976) Biochimie 58 601–610
[5] Guérin, M and Napias, C (1978) Biochemistry 17, 2510–2516
[6] Guérin, B and Guérin, M (1973) CR Acad Sci 276, 1503–1506
[7] Guérin B , Labbe, P and Somlo, M (1979) Methods Enzymol in press
[8] Velours J , Rigoulet, M and Guérin, B (1977) FEBS Lett 81, 18–22
[9] Rottenberg, H (1975) Bioenergetics 7, 61–74
[10] Somlo, M (1977) Arch Biochem Biophys 182, 518–524
[11] Nicholls,D G and Bernson, V S M (1977) Eur J Biochem 75, 601–612
[12] Tokumitsu Y and Ui, M (1973) Biochim Biophys Acta 292, 310–324
[13] Tokumitsu, Y and Ui, M (1973) Biochim Biophys Acta 292, 325–337